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Binding of folates to *Dictyostelium discoideum* cells. Demonstration of five classes of binding sites and their interconversion

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Studies of the binding of four folate derivatives to the cell surface of *Dictyostelium discoideum* indicate the existence of five types of sites. About 99% of the total number of binding sites (160 000 per cell) belongs to the 'non-selective' type, which recognizes folate, 2-deaminofolate and methotrexate with equal affinity. As judged by the kinetics of association and dissociation this class consists of two distinct subtypes; a high-affinity site, designated by A^H, and a low-affinity site A^L. Upon addition of ligand a number of the low-affinity sites is converted to the high-affinity state. Prolonged dissociation revealed the presence of extremely slowly dissociating sites. While the A-sites released bound ligand within 5 s, the slow (B) type yielded a half-time of about 6 min. This class (550 sites per cell) showed a clear selectivity for the four folates, with N_{10}-methylfolate being the best ligand. From the kinetics of association and dissociation it is concluded that the B-sites are interconvertible with another binding type. In addition a class of sites was detected, which binds N_{10}-methylfolate and folate with high affinity but 2-deaminofolate and methotrexate with approx. 100-fold lower affinity. Kinetic studies reveal that this C-class is also composed of two subtypes; a fast equilibrating site (within 1 s) designated as C^F, and a slower site C^S. It is proposed that before binding of ligand only C^F exists, while after binding this binding type is converted into C^S. At equilibrium more than 90% of the C-sites have attained the C^S state.

Introduction

In the vegetative stage cellular slime molds are free-living amoebae. Lack of food induces cell differentiation, during which the cells are synchronized and eventually aggregate by chemotactic movement. The multicellular aggregates will then form fruiting bodies containing spores that are resistant to unfavorable conditions. This asexual life cycle provides an excellent tool for studying intercellular communication. A point of much interest is the detection of extracellular signals by cell surface receptors and transduction of the information into the cell. Cyclic AMP (cAMP) and folic acid are well known signal compounds. Folic acid, like cAMP, evokes several cellular responses in *Dictyostelium discoideum*. Cells show oriented movement in a gradient of this compound [1]. A rapid transient increase in cGMP can be monitored intracellularly [2,3] and a slower transient elevation of cAMP is observed inside the cells as well as in their medium [4]. Repeated stimulation with folate is known to accelerate development to aggregation competence [5,6].

The cells possess a highly efficient signal degradation system, i.e. rapid enzymatic conversion of folic acid into 2-deaminofolic acid. This product is at least 1000–10 000-fold less active than folic acid as a chemoattractant [7] and elicits no detectable cGMP response at 10^{-4} M [8]. At a

lower rate 2-deaminofolic acid is further inactivated by a folate C_9-N_{10} cleaving enzyme, yielding lumazin-6-carboxaldehyde and *p*-aminobenzoylglutamate [9].

The presence of two distinct cell surface binding sites, with different ligand specificity with respect to folic acid and 2-deaminofolic acid [10] completes a system of two independent receptors with their own ligand degrading enzymes. (1) One receptor is 'folic acid-selective', i.e., folic acid is bound with > 100-fold higher affinity than 2-deaminofolic acid. Signal degradation for this receptor is achieved by deamination. (2) The second receptor is 'folic acid/2-deaminofolic acid non-selective', i.e. binds 2-deaminofolic acid and folic acid with equal affinity. The signal for this receptor is degraded by C_9-N_{10} cleavage of the folates.

Recently, three types of cAMP binding sites were demonstrated in *D. discoideum* using non-equilibrium binding studies and drugs modifying the proportioning between these types of sites [11].

For the oligopeptide receptor on leukocytes it has been proposed that distinct classes of sites transduce the signal of ligand binding to different cellular responses [12,13]. In the present report we describe several classes of folate binding sites and propose incubation conditions that allow specific determination of the ligand binding to each class. This knowledge was then used to study the relationship between binding to the distinct classes of sites and the chemotactic response. A report of this study is presented in the accompanying paper [14].

Materials and Methods

Cultures. Dictyostelium discoideum NC4(H) was cultivated together with *Escherichia coli* 281 on a solid medium containing 3.3 g peptone, 3.3 g glucose, 4.5 g KH_2PO_4, 1.5 g $Na_2HPO_4 \cdot 2H_2O$ and 15 g agar per liter. Cells were harvested after 40 h of growth at 21°C, before clearing of the bacterial lawn occurred. The cells were then washed three times by centrifugation at $150 \times g$ for 4 min in 10 mM sodium/potassium phosphate buffer (pH 6.5) at 5°C. The cell density was adjusted to $5 \cdot 10^7$ per ml and the suspension was aerated at 0°C for 10 min before use in binding assays.

Chemicals. The radioligands [7,3′,5′-^{3}H] methotrexate (15 Ci/mmol) and [7,9,3′,5′-^{3}H]folic acid (15 Ci/mmol) were purchased from the Radiochemical Centre (Buckingshamshire, U.K.). 2-Deamino[7,9,3′,5′-^{3}H]folic acid was prepared enzymatically from [^{3}H]folic acid as reported previously [10]. Unlabeled 2-deaminofolic acid was prepared by enzymatic deamination of folic acid (BDH Biochemicals, Poole, U.K.) as described in Ref. 7. N_{10}-Methyl[7,3′,5′-^{3}H]folic acid and unlabeled N_{10}-methylfolic acid were prepared by alkaline hydrolysis from [7,3′,5′-^{3}H]methotrexate and unlabeled methotrexate [15]. The products were purified by RP-18 HPLC and identified by their ultraviolet spectra [16]. Methotrexate (L-amethopterin) was obtained from Sigma Co. (St. Louis, MO). 8-Azaguanine was purchased from Fluka A.G. (Buchs SG, Switzerland). Silicon oil AR 20 and AR 200 were from Wacker Chemie (München, F.R.G.). The purity of all labeled and unlabeled folates was routinely checked by RP-18 HPLC. If necessary the compounds were purified by HPLC. Since folic acid and 2-deaminofolic acid are extremely light sensitive, samples were kept in the dark as much as possible. Methotrexate is easily hydrolyzed to N_{10}-methylfolic acid (which is over 100-fold more active in some cases). Purification by HPLC yielded less than 0.05% of N_{10}-methylfolic acid in the methotrexate preparation. The high deaminase activity of *D. discoideum* cells was inhibited by 0.33 mM 8-azaguanine, resulting in less than 5% degradation during the incubations.

Binding assay. Cells were prepared as described above. Final density in the incubation mixture was $3.3 \cdot 10^7$ cells/ml. The sample volume was 150 µl, which was layered on top of 180 µl silicon oil (AR 20 : AR 200 = 11 : 4) and 10 µl 10% sucrose. All incubations were performed at 0°C and terminated by centrifugation of the cells through the oil layer at $10\,000 \times g$ for 15 s. For this purpose a swing-out rotor was developed for the Eppendorf centrifuge. The tubes were frozen in liquid nitrogen and the tips containing the sucrose drops and cell-pellets were cut off. Radioactivity was measured after dissolving the pellets in 1.5 ml Instagel (Packard). Less than 5% of the radioligand was degraded by C_9-N_{10} cleavage or deamination during the incubations. Blank values were obtained in the presence

of excess (0.2 mM) unlabeled ligand and subtracted from all data, unless indicated otherwise. The standard error in all determinations was 5%. *E. coli* 281 at a density of $5 \cdot 10^7$ ml^{-1} did not bind detectable amounts of [^{3}H]folic acid as judged by this assay. The density of bacteria in the washed cell suspension was always lower than $5 \cdot 10^6$ ml^{-1}. In addition, *D. discoideum* cells (strain AX 2) which were grown axenically, showed binding properties similar to those of cells grown on bacteria. Therefore, bacteria should not be responsible for any folate binding. All five binding types were also detected in similar amounts on isolated *D. discoideum* membranes. It is thus unlikely that the minor binding types (B- and C-sites) result from broken or damaged cells, while only the principal sites (A-sites) are localized on the intact cell surface.

Technique for incubations shorter than 4 s. For rapid association experiments 100 µl cell suspension was layered on the silicon oil, while the reaction tube was already in the centrifuge. Incubation was started by pipetting 50 µl harvesting buffer containing radioligand onto the cell suspension. In the case of short dissociation times, 100 µl cell suspension was mixed with 50 µl incubation mix and layered on top of the oil. After the desired incubation time dissociation was started by addition of 3 µl 10^{-2} M unlabeled ligand. For the purpose of rapid pipetting and mixing a membrane air pump was used to blow the 50 µl or 3 µl aliquot into the cell suspension and immediately afterwards air bubbles were blown through the mixture without disturbing the oil layer. This procedure took less than 0.5 s; consequently centrifugation was started at the desired time point (1–4 s) in order to terminate the incubation (or dissociation) and separate bound from unbound ligand.

Results

Binding of methotrexate and 2-deaminofolic acid reveals A-sites

The kinetics of association of methotrexate, 2-deaminofolic acid and folic acid at several concentrations are shown in Fig. 1a. From the logarithmic plots of these data (Figs. 1b, 1c) it is obvious that association is biphasic. Within 2 s a fast exchanging site is at equilibrium, while after that time point a slower first order association process is observed. The rate constant of association (i.e. the slope in Fig. 1b) of 5 nM methotrexate, or 3.5 nM 2-deaminofolic acid and 3.5 nM folic acid, is 0.07 s^{-1}. Higher ligand concentrations (670 nM) yield an on-rate of 0.12 s^{-1}.

If the biphasic association is caused by the presence of two independent classes of sites, mainly the fast exchanging sites will be occupied after

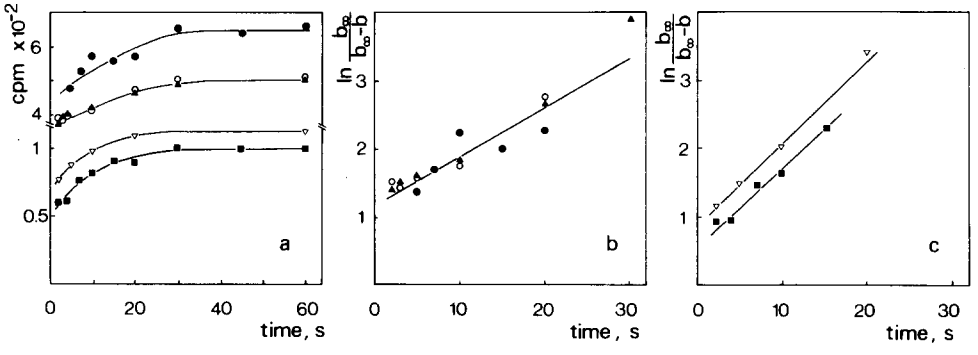

Fig. 1. Kinetics of association. (a) Time-course of association of [^{3}H]methotrexate at 5 nM (●), and at 671 nM (5 nM [^{3}H]methotrexate plus 666 nM unlabeled methotrexate) (▽); 2-deamino[^{3}H]folic acid at 3.5 nM (▲) and at 671 nM (3.5 nM 2-deamino[^{3}H]folic acid plus 666 nM unlabeled 2-deaminofolic acid) (■); [^{3}H]folic acid at 3.5 nM in the presence of 0.33 mM 8-azaguanine (○). Each data point is the mean of duplicate determinations in five independent experiments. (b) Semilogarithmic plot of the kinetics of association. 5 nM [^{3}H]methotrexate (●); 3.5 nM 2-deamino[^{3}H]folic acid (▲); 3.5 nM [^{3}H]folic acid (○). (c) Semilogarithmic plot of the kinetics of association at high ligand concentrations. 671 nM [^{3}H]methotrexate (▽); 671 nm 2-deamino[^{3}H]folic acid (■). *b* represents binding at the time points indicated; the equilibrium binding (b_∞) was determined after 60 s incubation.

short incubation times. As a result, dissociation after short association will reflect the off-rate of the fast exchanging sites. After longer incubations, also the slower equilibrating sites will be occupied. As a consequence the dissociation should be biphasic, reflecting release of ligand from both classes of sites. The results of such an experiment are presented in Fig. 2. The dissociation of [^{3}H]methotrexate was followed after either 4 s or 60 s of association. Apparently [^{3}H]methotrexate is released with a rate constant of 0.9 s^{-1}, irrespective whether the preincubation was for 4 s or 60 s. This result is incompatible with a model of two fixed classes of sites; the slowly associating binding component must represent a process different from binding to a slowly associating site. In order to obtain more information about this process, Scatchard analysis of the binding data after 2 s of incubation and at equilibrium was performed (Fig. 3). According to the kinetics of dissociation of [^{3}H]methotrexate, also association of this ligand should achieve at least 83% of the equilibrium value at 2 s of incubation, since

$$b_t = b_\infty(1 - e^{-(k_1 L + k_{-1})t}) \quad (1)$$

in which b_t represents bound ligand at a given time point, b_∞ is bound ligand at equilibrium, L is the ligand concentration, t is time, and k_1 and k_{-1} are the rate constants of association and dissociation, respectively. If $L \geq K_d$, association is faster than the rate of dissociation. Hence, all the data in Fig. 3 are obtained at binding equilibrium, though a distinct process occurs which requires approx. 30 s for equilibration. Apparently this process results in an increase of the affinity of the population of [^{3}H]methotrexate binding sites without changing the number of sites. At 2 s as well as 60 s the Scatchard plots are concave upward suggesting either binding site heterogeneity or negative cooperativity.

Fig. 4 shows the results of competition of [^{3}H]methotrexate binding by methotrexate, 2-deaminofolic acid, folic acid and N_{10}-methylfolic acid. The concentration yielding half-maximal inhibition (I_{50}) is similar for compounds modified in the pterin moiety: 100 nM for 2-deaminofolic acid, 130 nM for methotrexate and folic acid. Methylation of N_{10} resulted in a decreased affinity: 1 μM for N_{10}-methylfolic acid. These sites, which are non-selective for modification of the pterin moiety,

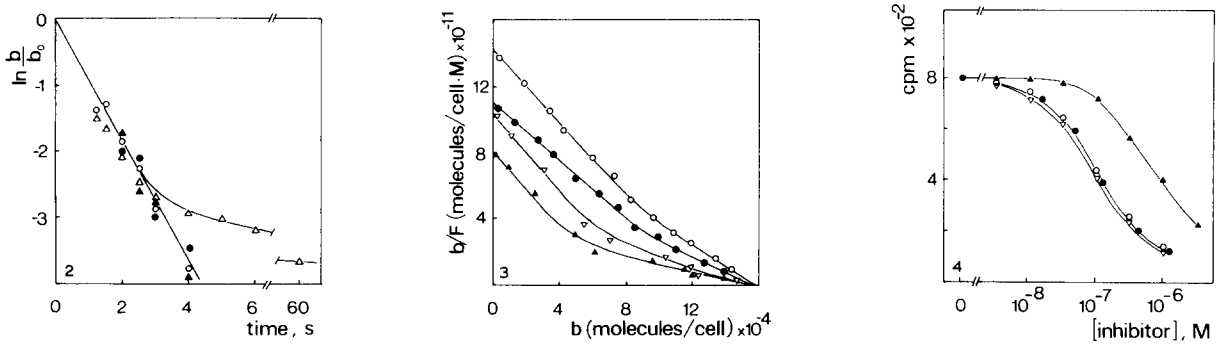

Fig. 2. Kinetics of dissociation of [^{3}H]methotrexate and 2-deamino[^{3}H]folic acid after the addition of 0.2 mM unlabeled ligand. Samples were preincubated with 5 nM [^{3}H]methotrexate for 4 s (●) or 60 s (○), or 5 nM 2-deamino[^{3}H]folic acid for 45 s (▲) or 60 s (△). b_0 was determined before the addition of excess unlabeled ligand after either 4 s or 60 s incubation with radioligand. The data are means of three independent experiments, in each of which incubations were in duplicate.

Fig. 3. Scatchard analysis of the binding of 2-deaminofolic acid and methotrexate at equilibrium (60 s) or pseudo-equilibrium (2 s). 2-Deamino[^{3}H]folic acid binding at 2 s (▽), at 60 s (○); [^{3}H]methotrexate binding at 2 s (▲), at 60 s (●). The data at 2 s were obtained in two independent experiments, those at 60 s in five experiments, in which all incubations were in duplicate.

Fig. 4. Competition of folates for the binding of 5 nM [^{3}H]methotrexate after 60 s incubation. All data were obtained in the presence of 0.33 mM 8-azaguanine. Competitors: methotrexate (●), folic acid (○), 2-deaminofolic acid (▽) and N_{10}-methylfolic acid (▲). Each data point is the mean of duplicate determination in two independent experiments.

will be designated as A-sites.

Since the Scatchard plot for methotrexate as well as 2-deaminofolic acid binding is only slightly curved, Lineweaver-Burk plots also should not deviate much from straight lines. Thus, analysis for competitive or non-competitive inhibition should be possible. Fig. 5 presents the results of such an experiment. Both competition of 2-deaminofolic acid for [^{3}H]methotrexate binding and competition of methotrexate for 2-deamino[^{3}H]folic acid binding were studied.

A Lineweaver-Burk plot of 2-deaminofolic acid competition for [^{3}H]methotrexate binding is shown in Fig. 5a; a replot of the abscissa intercepts is shown in Fig. 5b. Such a replot allows simple determination of the K_d values for the radioligand (ordinate intercept) and of the K_i value (inhibition constant) for the competitor (abscissa intercept). These two plots are arbitrarily interpreted as straight lines, though a slight curvature is expected. The straight lines, however, allow graphical determination of binding constants. Obviously, 2-deaminofolic acid and methotrexate are competitive ligands. The apparent K_d value for [^{3}H]methotrexate is 150 nM, which is very close to the apparent K_i for methotrexate: 145 nM (not shown). The same was obtained for 2-deaminofolic acid; the apparent K_d and K_i values were both 110 nM. Scatchard analysis, as shown in Fig. 3, resulted in similar $K_{0.5}$ values: 110 nM for 2-deaminofolic acid, 140 nM for methotrexate. Apparently, 2-deaminofolic acid and methotrexate compete for identical binding sites. In addition,

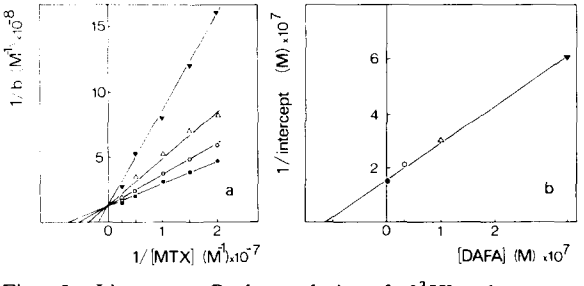

Fig. 5. Lineweaver-Burk analysis of [^{3}H]methotrexate ([^{3}H]MTX) binding data. (a) Inhibition of [^{3}H]methotrexate binding by 2-deaminofolic acid at various concentrations: 0 nM (●), 33 nM (○), 100 nM (△) and 333 nm (▼). (b) Replot of the reciprocal abscissa intercepts from panel (a) vs. the concentrations of 2-deaminofolic acid (DAFA). The symbols are as in panel (a). All data were determined in triplicate.

the kinetics of dissociation of 2-deamino[^{3}H]folic acid (Fig. 2) resemble those of [^{3}H]methotrexate (Fig. 2). The k_{-1} value is 0.9 s^{-1}, which is the same as for methotrexate. However, after more than 1 min of dissociation still some binding of 2-deaminofolic acid is detected, in contrast to methotrexate. The amount of this apparently more slowly dissociating 2-deaminofolic acid binding is negligible in relation to the binding to the A-sites (3% at 5 nM 2-deamino[^{3}H]folic acid). Therefore, this observation is not discussed in this section. The graphs in Fig. 3 indicate that the number of A-sites per cell, as obtained with 2-deaminofolic acid, is equal to the number as obtained with methotrexate. These observations confirm the hypothesis that 2-deaminofolic acid and methotrexate bind to identical binding sites. 2-Deaminofolic acid binds with a slightly higher affinity to these sites than does methotrexate.

As observed with 2-deaminofolic acid, also folic acid and N_{10}-methylfolic acid appear to be competitive inhibitors for [^{3}H]methotrexate at the A-sites (data not shown).

Residual binding after 60 s dissociation reveals B-sites

As stated in the previous section, ligand bound to A-sites exchanges relatively fast: after 60 s dissociation no residual binding of the original 600 cpm (at about 5 nM [^{3}H]methotrexate or 2-deamino[^{3}H]folic acid) is expected according to Eqn. 2.

$$b_t = b_0 e^{-k_{-1} \cdot t} \quad (2)$$

This was indeed observed for methotrexate, but not for 2-deaminofolic acid. After 60 s dissociation, 6 ± 3 cpm 2-deamino[^{3}H]folic acid was still retained above a blank value of 50 ± 2 cpm (Fig. 2). In order to improve the ratio between the binding and the blank value, dissociation was performed by 8-fold dilution of the samples in buffer containing an excess of unlabeled ligand and the cell density was increased. The blank value was thus lowered to 13 ± 1.5 cpm. Fig. 6a presents the kinetics of dissociation of 2-deaminofolic acid, folic acid and N_{10}-methylfolic acid on a minute time scale. All three compounds appear to be released with the same velocity: k_{-1} is $2 \cdot 10^{-3}$ s^{-1}. Ap-

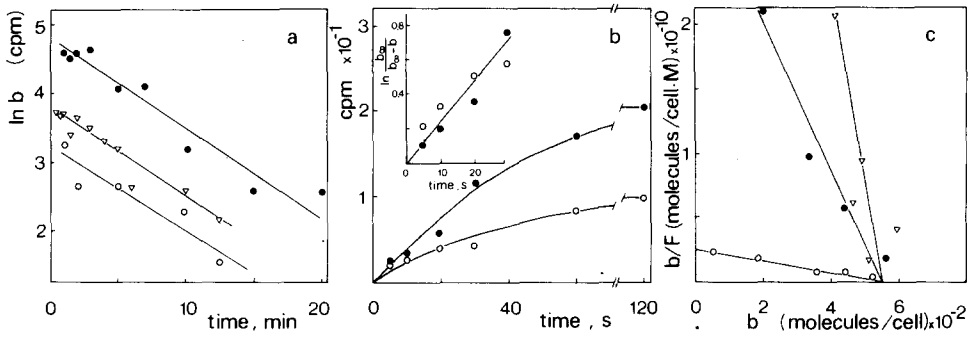

Fig. 6. (a) Kinetics of dissociation after 8-fold dilution with assay buffer containing 25 μM unlabeled ligand. Per sample $1.5 \cdot 10^7$ cells were incubated with 20 nM [^{3}H]folic acid (●), 20 nM 2-deamino[^{3}H]folic acid (○) or 5.5 nM N_{10}-methyl[^{3}H]folic acid (▽) for 120 s. Consequently dissociation was initiated by addition of excess unlabeled ligand and the volume was 8-fold enlarged in order to lower the blank value. During the incubation 0.33 mM 8-azaguanine was present. (b) Time-course of 2-deaminofolic acid association as determined after 60 s dissociation. The chase conditions were as described for panel (a). 20 nM 2-deamino[^{3}H]folic acid (●) or 287 nm 2-deamino[^{3}H]folic acid (20 nM 2-deamino[^{3}H]folic acid plus 267 nM unlabeled) (○) was incubated with $1.5 \cdot 10^7$ cells per sample and 0.33 mM 8-azaguanine for 5–120 s. After the desired incubation time, the binding was chased for 60 s. Inset: semilogarithmic plot of the data, b_∞ was determined after 120 s of association followed by 60 s of dissociation. (c) Scatchard analysis of equilibrium binding after a 60 s chase procedure as described for panel (a). Association was for 120 s with $1.5 \cdot 10^7$ cells per sample and 0.33 mM 8-azaguanine. The residual binding was corrected for a loss of 11% during the chase procedure [^{3}H]folic acid (●); N_{10}-methyl[^{3}H]folic acid (▽); 2-deamino[^{3}H]folic acid (○). Each data point is a mean of duplicate determination in three experiments.

parently, as for 2-deaminofolic acid, also the binding of folic acid and N_{10}-methylfolic acid to other classes of sites is negligible after 60 s of dissociation, since no biphasic dissociation behavior is observed. According to Eqn. 2 89% of the original binding to this slowly dissociating class is recovered after 60 s of dissociation. Thus it is possible to study binding to this type of binding site. In Fig. 6b the kinetics of association are shown. Obviously, the kinetics of association are independent of the ligand concentration. The apparent first order rate constant is 0.024 s^{-1} for 2-deaminofolic acid.

Since the binding is at equilibrium after approx. 120 s of incubation, a Scatchard analysis of the binding of folic acid, N_{10}-methylfolic acid and 2-deaminofolic acid was performed (Fig. 6c). Folic acid and N_{10}-methylfolic acid bind with high affinity, the apparent K_d values are 17 and 4 nM, respectively, while for 2-deaminofolic acid an apparent K_d value of 240 nM was obtained. Methotrexate was not bound in detectable amounts. Furthermore, folic acid, N_{10}-methylfolic acid and 2-deaminofolic acid apparently bind to a similar number of binding sites: approx. 550 per cell.

In order to obtain more accurate information about the affinity of methotrexate for this type of binding sites, inhibition of [^{3}H]folic acid binding was determined as shown in Fig. 7. Half-maximal inhibition was achieved at 33 μM. In addition the potency of 2-deaminofolic acid and N_{10}-methylfolic acid to complete with [^{3}H]folic acid was monitored. As shown in Fig. 7 the I_{50} values are 400 nM for 2-deaminofolic acid and 10 nM for

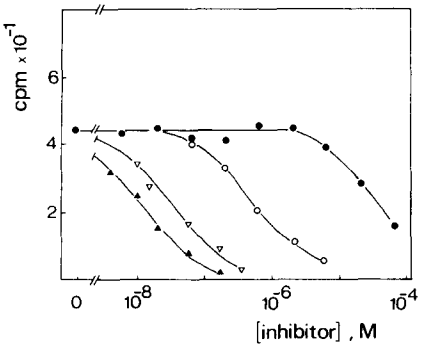

Fig. 7. Competition of folates for the binding of 10 nM [^{3}H]folic acid, determined after a 60 s chase procedure as described for Fig. 6a. The binding was equilibrated for 120 s with $1 \cdot 10^7$ cells per sample in the presence of 0.33 mM 8-azaguanine. Competitors: methotrexate (●); 2-deaminofolic acid (○), folic acid (▽) and N_{10}-methylfolic acid (▲). The data were determined in duplicate in five experiments.

N_{10}-methylfolic acid. The low affinity of methotrexate and the I_{50} values for 2-deaminofolic acid and N_{10}-methylfolic acid are in agreement with the absence of [^{3}H]methotrexate binding and the apparent K_d values of 2-deaminofolic acid (240 nM) and N_{10}-methylfolic acid (4 nM) for these slowly dissociating sites.

The class of slowly dissociating folate binding sites will be addressed as B-sites.

Binding of N_{10}-methylfolic acid and folic acid in the presence of excess amounts of 2-deaminofolic acid reveals C-sites

The folate binding sites, as described in the previous sections, have in common that the dissociation constants (or inhibition constants) of 2-deaminofolic acid all are in the range of approx. 100 nM to approx. 300 nM. If, however, competition of 2-deaminofolic acid for the binding of [^{3}H]folic acid or N_{10}-methyl[^{3}H]folic acid is examined, a significant fraction of bound ligand may only be removed by unexpectedly high concentrations of 2-deaminofolic acid. As shown in Fig. 8a, inhibition of 18.5 nM N_{10}-methyl[^{3}H]folic acid by 2-deaminofolic acid is clearly biphasic, 44% of the binding is inhibited with an I_{50} of 100 nM, the remaining 56% with an I_{50} of 45 μM. Inhibition constants may be related to I_{50} values according to Eqn. 3, provided that inhibition is competitive.

$$K_i = I_{50} \cdot \frac{K_d}{K_d + L} \qquad (3)$$

It has already been mentioned that N_{10}-methylfolic acid binds with low affinity to the A-sites. An I_{50} value of 1 μM was obtained for competition with 5 nM [^{3}H]methotrexate at the A-sites. Using Eqn. 3, it may be calculated that 2-deaminofolic acid should compete for the binding of 18.5 nM N_{10}-methyl[^{3}H]folic acid at the A-sites, with an apparent I_{50} of 110 nM. Indeed, one of the observed values fits this predicted I_{50} value. Binding of N_{10}-methylfolic acid to the B-sites will be inhibited by 2-deaminofolic acid with an I_{50} of about 360 nM; the contribution of the B-sites to the total binding is very low (approx. 5%). Therefore, these sites will not affect the data in Fig. 8a. The second I_{50} value (45 μM) should reflect binding of N_{10}-methylfolic acid to a distinct class of sites with a relatively low affinity for 2-deaminofolic acid. Fig. 8b is a Lineweaver-Burk plot of competition of 2-deaminofolic acid for N_{10}-methyl[^{3}H]folic acid binding to this new class of sites. The binding of labeled N_{10}-methylfolic acid to A-sites was prevented by addition of at least 2.7 μM 2-deaminofolic acid . As is clear from Fig. 8a, this concentration will inhibit the majority of N_{10}-methylfolic acid binding to the A-sites, while only a slight inhibition of the binding of N_{10}-methylfolic acid to the other class of sites is expected. This class of sites with a low affinity for 2-deaminofolic acid will be addressed by C-sites. Fig. 8b suggests that 2-deaminofolic acid is a competitive inhibitor of N_{10}-methylfolic acid bind-

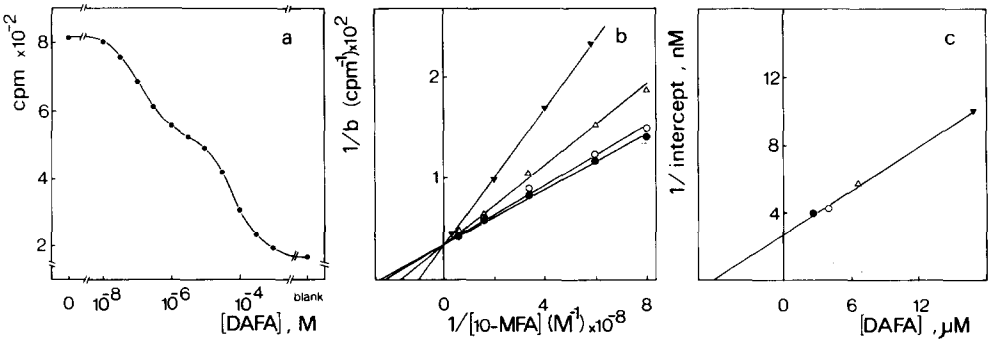

Fig. 8. (a) Inhibition of binding of 18.5 nM N_{10}-methyl[^{3}H]folic acid by 2-deaminofolic acid (DAFA). Incubation was for 60 s in the presence of 0.33 mM 8-azaguanine. These data were not corrected for the blank value as obtained in the presence of 0.2 mM N_{10}-methylfolic acid (10-MFA). The data are means of determination in duplicate. (b) Lineweaver-Burk analysis of the inhibition of N_{10}-methyl[^{3}H]folic acid binding in the presence of 0.33 mM 8-azaguanine by various concentrations of 2-deaminofolic acid: 2.7 μM (●); 4.1 μM (○); 6.7 μM (△) and 17 μM (▼). The data were determined in duplicate in three experiments. (c) Replot of the reciprocal abscissa intercepts from panel (b) vs. the concentrations of 2-deaminofolic acid. The symbols are as in panel (b).

ing to the C-sites. Apparently, no interference by binding to A-sites has occurred, since also higher 2-deaminofolic acid concentrations yield the same maximal binding level (ordinate intercept). A replot (Fig. 8c) of the abscissa intercepts of Fig. 8b yields the dissociation constant for N_{10}-methylfolic acid (2.8 nM, ordinate intercept) and the inhibition constant for 2-deaminofolic acid (abscissa intercept, 6.3 μM). These data suggest that the C-sites obey Michaelis-Menten kinetics. Though 2-deaminofolic acid at 3.3 μM slightly competes with N_{10}-methyl[^{3}H]folic acid, this 2-deaminofolic acid concentration was used during further investigations in order to prevent binding of N_{10}-methyl[^{3}H]folic acid to A-sites (and B-sites). The fact that 2-deaminofolic acid is present at a concentration which slightly inhibits N_{10}-methylfolic acid binding, should result in an apparent dissociation constant for N_{10}-methylfolic acid that is higher than the real value: 4.0 nM instead of 2.8 nM (Fig. 8c). The k_{-1} value, obtained in association experiments, will be increased proportional to the K_d value. The k_1 value should be unaffected.

Fig. 9a shows the kinetics of association of various concentrations of N_{10}-methylfolic acid. An observation that was already used for the preceding experiments, is that binding equilibrium is reached within 60 s. A semilogarithmic plot of the association process (Fig. 9b) is clearly biphasic, suggesting that association is composed of a fast and a slower component. At the earliest time point that could be achieved (1 s) the fast component was already in equilibrium. After 1 s, the binding increased with apparently first order kinetics. Because of the widely different rate constant of the two association components, two experiments were designed to measure both processes separately. (1) After various incubation times, bound ligand was (partially) chased for 6 s with excess unlabeled ligand. The aim of this experiment was to monitor the association rate to the slower type of site. The faster sites apparently reach equilibrium within 1 s, and should not retain bound ligand after a 6-s chase. (2) The rate of dissociation was determined after equilibrium binding as well as after only 4 s of association. Assuming that association proceeds to faster and slower sites, mainly the faster sites will be occupied after 4 s of association, since these are already at equilibrium, while the slower sites just start to be occupied. After longer association times also the slower sites will be occupied. Fig. 9b shows the rate of association, with and without the 6-s chase procedure. As expected, the biphasic behavior is absent after the chase procedure. Furthermore, the two lines are parallel, indicating identical rate constants. Using the chase procedure, association to only the slower binding type seems to be measured. Fig. 9c presents the kinetics of association of various ligand concentrations, as obtained using the chase procedure. A

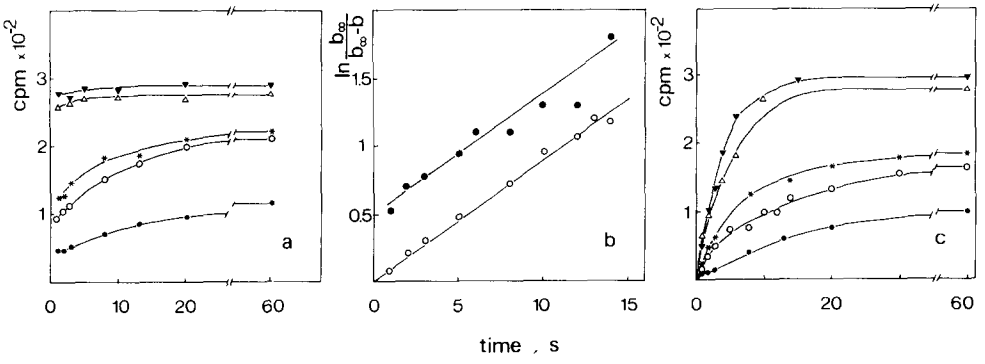

Fig. 9. (a) Kinetics of association of N_{10}-methyl[^{3}H]folic acid as determined in the presence of 3.3 μM 2-deaminofolic acid and 0.33 mM 8-azaguanine. Concentrations of N_{10}-methyl[^{3}H]folic acid: 1.85 nM (●); 3.7 nM (○); 5.5 nM (*); 27.6 nM (△) and 55 nM (▼). (b) Semilogarithmic plot of the kinetics of association of 3.7 nM N_{10}-methyl[^{3}H]folic acid (●). The conditions were as described for panel (a). Identical samples were treated with a 6-s chase procedure by addition of 0.2 mM unlabeled N_{10}-methylfolic acid after the desired association time (○). (c) Kinetics of association of various concentrations of N_{10}-methyl[^{3}H]folic acid, measured after a 6-s chase procedure as described for panel (b). The incubation conditions and symbols are as in panel (a). The data were corrected for a 21% loss of the original binding during 6 s of dissociation. The data were obtained in duplicate in five independent experiments.

semilogarithmic plot of these data yields linear relationships for all ligand concentrations that were tested (Fig. 10). Clearly, the rate constant of association to the slower site is dependent of the ligand concentrations.

Fig. 11 shows the results of the second approach: the rate of dissociation after short incubation (4 s) and after equilibrium binding (60 s). As expected, dissociation proceeded faster after 4 s of association than after 60 s. This suggests that association first occurs to faster dissociating sites and that longer association is required to occupy the slower dissociating sites. The k_{-1} value of the faster site could not be determined, since this type had already completely released its ligand within 4 s. As association to this site proceeds within 1 s, a minimum value for k_{-1} may be proposed: 2 s^{-1} (i.e. 86% of the equilibrium binding is reached at 1 s). The rate constant for the slower site is 0.04 s^{-1}. This value allows calculation of the binding recovery after the 6-s chase procedure: 79%. (The data in Fig. 9c were corrected for 21% loss in binding). From now on the faster sites will be designated by C^F, the slower sites by C^S.

Fig. 12 presents a replot of the association rates to C^S (from Fig. 10) versus the ligand concentration. Michaelis-Menten kinetics should result in a linear relationship; the K_d value may be determined from the abscissa intercept, k_{-1} from the ordinate intercept and k_1 may be determined from the slope. The three constants are: K_d 4.0 nM, k_{-1} 0.048 s^{-1} and k_1 $1.2 \cdot 10^7$ $M^{-1} \cdot s^{-1}$. These values are in exact agreement with data from equilibrium binding (Fig. 8c): after correction for the effect of 2-deaminofolic acid, the K_d value is 2.8 nM and k_{-1} is 0.034 s^{-1}. So, equilibrium binding mainly reflects the C^S sites: more than 90% of the equilibrium binding is contributed by C^S sites (Fig. 11). Apparently, an upper limit for the rate of association to C^S exists, since at concentrations higher than 10 nM N_{10}-methyl[^{3}H]folic acid a plateau is reached. A possible cause for this observation will be discussed later.

A piece of information, which is still missing, is the affinity of the C^F sites for N_{10}-methylfolic acid. This may be determined by studying binding at a time point, at which binding to C^F is in equilibrium, while no or little binding has occurred to C^S sites. As mentioned before, this may be done by incubating for only 2 s. However, at higher ligand concentrations the rate of occupation of C^S is 0.2 s^{-1} (upper limit), yielding 33% of the equilibrium binding to C^S at 2 s. This will cause overestimation of the actual binding to C^F. How-

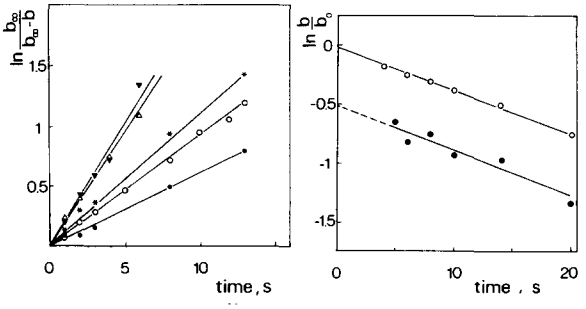

Fig. 10. Semilogarithmic plot of the association kinetics of N_{10}-methyl[^{3}H]folic acid, measured after a 6-s chase. Concentrations of labeled N_{10}-methylfolic acid: 1.85 nM (●); 3.7 nM (○); 5.5 nM (*); 27.6 nM (△) and 55 nM (▼). The data were calculated from Fig. 9c, b_∞ was determined after 60 s of incubation.

Fig. 11. Kinetics of dissociation of N_{10}-methyl[^{3}H]folic acid after association of 5.5 nM N_{10}-methyl[^{3}H]folic acid in the presence of 3.3 μM 2-deaminofolic acid and 0.33 mM 8-azaguanine for 4 s (○) or 60 s (○). b_0 was determined without addition of excess (0.2 mM) N_{10}-methylfolic acid. The data points are means of duplicate determinations in two experiments.

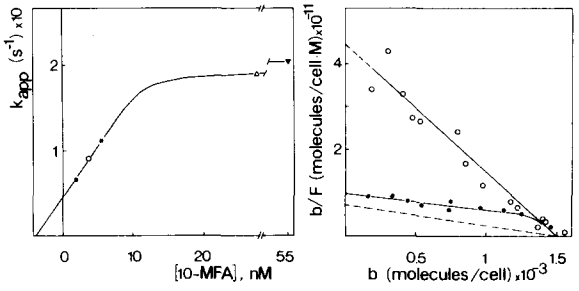

Fig. 12. Replot of the apparent rate constants of association (k_{app}), i.e. the slopes from Fig. 10 vs. the concentration of N_{10}-methyl[^{3}H]folic acid: 1.85 nM (●); 3.7 nM (○); 5.5 nM (*); 27.6 nM (△) and 55 nM (▼).

Fig. 13. Scatchard analysis of the binding of N_{10}-methyl[^{3}H]folic acid at equilibrium (60 s, (○)) and pseudo-equilibrium (2 s (●)). Incubations were done in the presence of 3.3 μM 2-deaminofolic acid and 0.33 mM 8-azaguanine. The dashed line was obtained after correction of the binding at 2 s (mainly C^F sites) for the contribution of C^S sites. The data were obtained in duplicate in three independent experiments.

TABLE I

BINDING PARAMETERS FOR FOLIC ACID (FA), N_{10}-METHYLFOLIC ACID (10-MFA), 2-DEAMINOFOLIC ACID (DAFA) AND METHOTREXATE (MTX) FOR THE DISTINCT CLASSES OF SITES

The binding parameters which are not mentioned in Results were determined by the following methods: (a) The K_d value of 2-deaminofolic acid and methotrexate for A^H and A^L sites as well as the numbers of A^H and A^L sites were derived by computer curve-fitting for the data in Fig. 3. (b) The K_d values for folic acid and N_{10}-methylfolic acid for the A^H and A^L sites were derived from competition with [^{3}H]methotrexate binding. It was assumed that these ligands would induce a change in the distribution between A^H and A^L similar to methotrexate and 2-deaminofolic acid, and that their selectivity between A^H and A^L would be similar to that of 2-deaminofolic acid and methotrexate. (This is affirmed by the observation, that the competition curves in Fig. 7 are parallel.) (c) The dissociation rate of folic acid from the A sites was determined by monitoring the release of [^{3}H]folic acid in a chase experiment. The data were corrected for contribution of B and C^S sites (only 10% of the equilibrium binding). (d) The dissociation of N_{10}-methylfolic acid from the A sites could not be measured accurately as a result of the large contribution of B and C^S sites (about 60%). Therefore, the dissociation of 2-deamino-N_{10}-methyl[^{3}H]folic acid was studied. This compound was synthesized enzymatically from N_{10}-methyl[^{3}H]folic acid, similarly to 2-deamino[^{3}H]folic acid (cf. chemicals). The rationale behind this is, that 2-deaminofolic acid has a k_{-1} value equal to that of folic acid; thus 2-deamino-N_{10}-methylfolic acid might yield a value identical to N_{10}-methylfolic acid (if the molecular modifications are independent). For 2-deamino-N_{10}-methylfolic acid the contribution of the B sites to the total binding amounted to less than 10%. The data were corrected for this amount. (e) The affinity of methotrexate for C^S was determined by competition for N_{10}-methylfolic acid binding at equilibrium. The obtained I_{50} value was transformed to a K_i value according to Eqn. 3. Assuming competitive inhibition, K_i values are exchangeable with K_d values. (f) The dissociation constants of 2-deaminofolic acid and methotrexate for the C^F sites were obtained by competition with N_{10}-methyl[^{3}H]folic acid at 2 s of association. The presented values are not very accurate, since competition is measured to C^F as well as C^S. The contribution of C^S at 2 s of incubation with various competitor concentrations is not known, but in all cases lower than 33%. (g) The affinity of folic acid for C^F was measured using the same approach as for N_{10}-methylfolic acid. (h) Association rate constants (k_1) were calculated from K_d and k_{-1} values.

Ligand		FA	10-MFA	DAFA	MTX	Number of sites per cell		
						initially after ligand binding	at equilibrium	total
Binding type								
A^H	K_d (nM)	70 ± 20	700 ± 200	55 ± 15	70 ± 20			
	k_{-1} (s^{-1})	0.9 ± 0.1	≥ 2	0.9 ± 0.1	0.9 ± 0.1	28000 ± 2500	80000 ± 3000	
	k_1 ($M^{-1}\cdot s^{-1}$)($\times 10^{-7}$)	1.3 ± 0.4	≥ 0.3	1.6 ± 0.5	1.3 ± 0.4			160000 ± 16000
A^L	K_d	450 ± 50	4500 ± 500	370 ± 60	450 ± 50			
	k_{-1}	0.9 ± 0.1	≥ 2	0.9 ± 0.1	0.9 ± 0.1	132000 ± 2500	80000 ± 3000	
	k_1 ($\times 10^{-6}$)	2.0 ± 0.3	≥ 4.4	2.4 ± 0.5	2.0 ± 0.3			
A^L-A^H conversion, k (s^{-1})				0.1 ± 0.03				
Binding type								
B	K_d	17 ± 5	4 ± 2	240 ± 80	20000			
	k_{-1} ($\times 10^3$)	2 ± 0.5	2 ± 0.5	2 ± 1	n.d.	0	550	550 ± 110
	k_{on} ($\times 10^3$)	n.d.	n.d.	24 ± 2	n.d.			
C^F	K_d	50 ± 10	20 ± 2	11000	25000			
	k_{-1}	≥ 2	≥ 2	n.d.	n.d.	≥ 1350	≤ 50	
	k_1 ($\times 10^{-8}$)	≥ 0.4	≥ 1	n.d.	n.d.			1500 ± 150
C^S	K_d	15 ± 3	2.8 ± 0.02	6300 ± 500	6000 ± 700			
	k_{-1} ($\times 10^2$)	32 ± 0.3	3.7 ± 0.2	n.d.	n.d.	≤ 150	≥ 1450	
	k_1 ($\times 10^{-7}$)	2.1 ± 0.5	1.3 ± 0.1	n.d.	n.d.			
C^F-C^S conversion, k (s^{-1})				0.2 ± 0.02				

ever, at low ligand concentrations, e.g. 1.5 nM, 11% of the equilibrium binding to C^S will be found after 2 s. These percentages may be derived from Fig. 12 and since the equilibrium binding to C^S is known, the binding at 2 s may be corrected for the contribution by C^S. Fig. 13 shows the data of binding at equilibrium (to C^S), at 2 s, and a corrected graph for the actual binding to C^F (dashed line). As expected, C^F as well as C^S behave as simple Michaelis-Menten binding sites under these extreme conditions. The K_d value of C^S is 3.6 nM (2.6 nM, after correction for the effect of 3.3 μM 2-deaminofolic acid), which is close to the value as obtained by other methods. The K_d value of C^F is 20 nM. The number of C sites per cell amounts to about 1500.

These properties of N_{10}-methylfolic acid binding were checked by a study of the binding of [^{3}H]folic acid. As with N_{10}-methyl[^{3}H]folic acid the association process is biphasic: a fast component within 1 s and a slower process with a velocity of 0.22 s^{-1} (data not shown). After a short (4 s) chase procedure, the fast component is absent, while the slower process is still observed: 0.18 s^{-1}. Since binding should be at equilibrium within 30 s, Scatchard analysis of [^{3}H]folic acid binding was performed at that time point. Apparently, one binding site is observed, with a K_d value of 22 nM. The presence of 3.3 μM 2-deaminofolic acid results in a 1.43-fold increased k_d value (as with N_{10}-methylfolic acid). Thus, the actual K_d should be 15 nM. The number of binding sites per cell is 1500, as was also derived from N_{10}-methyl[^{3}H]folic acid binding. After equilibration, the kinetics of dissociation yield a k_{-1} value of 0.32 s^{-1}.

Because of the higher affinity of N_{10}-methylfolic acid compared to folic acid, and therefore more favorable binding-blank ratios for N_{10}-methyl[^{3}H]folic acid, this compound was used for studying the C sites. The binding parameters as presented above, are all summarized in Table I. In addition, the missing constants were determined as described in the legends to Table I. Table II shows the conditions that may be applied to determine

TABLE II

CONDITIONS FOR SEPARATE MEASUREMENTS OF THE DISTINCT BINDING CLASSES

FA, folic acid; DAFA, 2-deaminofolic acid; 10-MFA, N_{10}-methylfolic acid; MTX, methotrexate.

Type	Conditions	Calculated % of binding caused by other sites
A^H	5 nM [^{3}H]MTX, 60 s incubation	13% by A^L < 0.1% by B, C^F and C^S
A^L	670 nM [^{3}H]MTX, 2 s incubation	37% by A^H < 0.1% by B, C^F and C^S
B	5 nM [^{3}H]FA, 120 s incubation followed by 60 s dissociation (8-fold dilution in the presence of excess unlabeled FA)	20% by A^H and A^L < 0.1% by C^F and C^S
	after 120 s dissociation	< 0.1% by A^H, A^L, C^F and C^S
C^F	2 nM [^{3}H]10-MFA, in the presence of 3.3 μM DAFA, 2 s incubation	1% by A^H and A^L 1.5% by B 27% by C^S, up to 31% C^S at ligand concentrations higher than 30 nM
C^S	2 nM [^{3}H]10-MFA, in the presence of 3.3 μM DAFA, 60 s incubation (K_d and k_{-1} as obtained from equilibrium and association studies will be 1.43 times the actual values)	0.6% by A^H and A^L 1% by B 12% by C^F; less at higher ligand concentrations

binding to each type of site separately. Additionally, the calculated deviations as a result of the other sites are given.

Discussion

In the present report a detailed study of folate binding to *D. discoideum* cells has been described. Two approaches allowed the detection of five distinct classes of binding sites, (1) the use of four different radioligands: folic acid, N_{10}-methylfolic acid, 2-deaminofolic acid and methotrexate, (2) non-equilibrium binding studies. Two of these classes, referred to as A sites, do not discriminate between folic acid, 2-deaminofolic acid and methotrexate as ligands, while N_{10}-methylfolic acid is about 10-fold less potent as ligand. The difference between the two A types is found in the affinity of ligand binding. If binding of 2-deamino[^{3}H]folic acid or [^{3}H]methotrexate is monitored, only the A sites will be observed and slightly curved Scatchard plots will be obtained. This curvature may be caused either by heterogeneity of the A class or by negative cooperativity. A previous report on 2-deamino[^{3}H]folic acid binding [17] is in agreement with the present results, while [^{3}H]methotrexate binding [18] was reported to yield a linear Scatchard plot. The curvature might have been obscured by the larger experimental errors in those experiments than in ours. As observed in our hands, the centrifugation assay employing polyethylene glycol instead of silicon oil, is less accurate.

Binding of folic acid, 2-deaminofolic acid or methotrexate (equilibrium within 2 s) is followed by a slower process (half-time 7–10 s), which is visible as an increase in affinity of the receptors for these ligands, without affecting the number of binding sites. Recently, we have shown that this affinity modulation is absent in isolated membranes from *D. discoideum,* but that it may be induced by guanine nucleotides [19]. Probably, high-affinity A-sites (A^H) and low-affinity H-sites (A^L) exist, which may interconvert upon ligand binding. If the Scatchard plots for methotrexate and 2-deaminofolic acid in Fig. 3 are curve-fitted for a two-class binding site model, the parameters in Table I are obtained. These data suggest that initially the radioligands bind to a heterogeneous receptor population consisting of approx. 16% A^H (K_d 50–70 nM) and approx. 84% A^L (K_d 370–450 nM). Consequently part of the A^L-sites is converted to the A^H form, yielding 50% A^H and 50% A^L.

As reported in Ref. 18, the potency of folic acid to act as a competitor of [^{3}H]methotrexate binding was about 30-fold lower than that of methotrexate. This is in contradiction with the present data suggesting that folic acid and methotrexate are equipotent. A simple explanation for this was not found. It may, however, be the result of two plausible processes. At the one hand complete deamination of the added folic acid is expected, when the deaminase activity is not inhibited. The newly formed 2-deaminofolic acid should, however, effectively compete for [^{3}H]methotrexate binding. At the other hand, [^{3}H]methotrexate is readily hydrolyzed to N_{10}-methyl[^{3}H]folic acid. The latter will bind to sites of the C type, for which 2-deaminofolic acid will only compete at concentrations much higher than required for competition at the A sites.

A third type of binding site (B) is recognized by its relatively slow kinetics of dissociation. Also the specificity for the four ligands is different from that of the A sites: folic acid and N_{10}-methylfolic acid are bound with high affinities, 17 nM and 4 nM, respectively. 2-Deaminofolic acid is bound less effectively (K_d = 240 nM), while methotrexate is a more than 1000-fold weaker ligand compared to folic acid. Two observations suggest that these compounds bind to identical sites. (1) Scatchard analysis yields the same number of slowly dissociating binding sites per cel for each of the ligands. (2) The ligand specificity of the slowly dissociating sites, as obtained from binding of the labeled ligands (Fig. 6c), is similar to the specificity as obtained by competition of the four ligands for the binding of [^{3}H]folic acid to these sites (Fig. 7). The rate of association to this binding site is independent of the ligand concentration. In addition, the rate of association is about 12-fold higher than the dissociation rate. These properties are in conflict with the law of mass action. This may indicate the B site to be more complicated than a single fixed class. Scatchard analysis and kinetics of dissociation, however, apparently reveal only one binding type at equilibrium. If association would proceed

to this type, the kinetics of association should have been slower than was observed. Also, the rate should have been dependent on the ligand concentration (provided that according to Eqn. 1 the concentration is equal to or higher than the dissociation constant). Since this was not observed, association might take place to a more rapidly equilibrating type of site, which is then converted into the observed B sites. The velocity of conversion might be rate limiting and independent of the ligand concentration. Thus, the apparent association of ligand to the B sites might actually be the process of formation of (already occupied) B sites out of a more rapidly equilibrating type of site. At this moment no data are available about the identity of this hypothetical site.

In an earlier report the presence of binding sites with a high affinity for folic acid and a much lower affinity for 2-deaminofolic acid was already shown [1]. This binding type, now referred to as C sites, shows properties significantly different from those presented in the preliminary study. The K_d value is now 15 nM, instead of 700 nM. However, the latter value was obtained in the presence of 20 μM 2-deaminofolic acid (3.3 μM 2-deaminofolic acid in this study). The relationship between the apparent K_d value obtained in the presence of competitor, and the real K_d is given by

$$K_d \text{ app} = K_d\left(1 + \frac{I}{K_i}\right) \quad (4)$$

in which I is the concentration of competitive inhibitor. After correction according to this equation, the actual K_d value becomes 165 nM. This is still significantly different from the K_d value of 15 nM as obtained in the present investigation. Also no explanation was found for the difference in number of sites per cell: 100000 in the earlier report, but 1500 presently. The present data, however, are highly reproducible in more than 30 experiments.

At a first glance the C-sites are composed of two kinetically distinct subtypes: C^F and C^S. However, closer examination of the data in Figs. 9, 10 and 11 suggests a binding site model different from a simple system with two fixed classes. Several observations are incompatible with such a fixed class model. (1) The binding increments contributed by C^S in Fig. 9a are significantly smaller than the binding levels to C^S in the 6 s chase experiment in Fig. 9c. (2) Even when the total binding appears to be at equilibrium (e.g. 55 nM N_{10}-methylfolic acid in Fig. 9a), binding to C^S, as measured in the chase experiment, still increases (Fig. 9c). (3) The rate of association to C^S is dependent of the ligand concentration only up till 27.6 nM. Above that value no further increase is observed (Fig. 10). (4) Dissociation after equilibration with e.g. 5.5 nM N_{10}-methylfolic acid suggests 97% of the binding to occur to C^S sites and 3% to C^F sites (Fig. 11). In contrast, association of 5.5 nM N_{10}-methylfolic acid suggests 57% to C^S and 43% to C^F sites (Fig. 9a). A plausible explanation for point 4 is, that initially more C^F sites are present than after longer incubation. Point 2 suggests that binding may be constant, though binding to C^S increases. Thus, an increasing binding to C^S may be accompanied by a decreasing binding to C^F. As a result, total binding may be constant. Since this was observed at saturating concentrations of N_{10}-methyl[^{3}H]folic acid (binding did not increase further, when the ligand concentration was raised from 27.5 nM to 55 nM), also the number of C sites per cell should be constant, while the number of C^F and C^S sites may change. Whether or not C^F sites are transformed to C^S sites, may be indicated by point 1. If part of the ligand eventually bound to C^S, did not associate to C^s (slow), but to C^F (fast), after which the occupied C^F site was transformed to a C^S site, the binding to C^S should be underestimated in Fig. 9a. However, in Fig. 9c, the actual amount of binding to C^S was determined after a chase of the binding to C^F. Thus, as mentioned in point 1, the binding level to C^S is expected to be lower in Fig. 9a than in Fig. 9c.

If association to C^S would obey Michaelis-Menten kinetics, Fig. 12 should have shown a linear relationship. However, at concentrations above 10 nM N_{10}-methyl[^{3}H]folic acid an upper limit for the rate of association is reached. A very plausible hypothesis may be, that this limit corresponds to the rate of transformation of C^F into C^S sites. Obviously, the apparent rate of occupation of C^S sites cannot exceed the rate of formation of these sites. The rate constant of this transformation may thus be 0.2 s^{-1}. It is not clear whether

low ligand concentrations induce complete conversion or not. Apparently, the lowest concentration of N_{10}-methylfolic acid that was used (0.5 nM) still induced maximal conversion, since a Scatchard plot (Fig. 13) is linear down till that concentration. Lower concentrations could not be used as a result of the low specific activity of N_{10}-methyl[^{3}H]folic acid (15 Ci · mmol^{-1}).

Generally, ligand induced increases in affinity are described by two models. (1) Positive cooperativity, in which an occupied site induces another site to enhance its binding affinity. In this model, site-site interactions occur [20]. (2) After occupation with ligand, the binding protein may attain an energetically more favorable conformation. This second state shows a higher ligand binding affinity. It is well known for vertebrate cells, that β-adrenergic ligands induce the receptor to interact with a GTP-binding protein, which in turn is reflected in an altered affinity between receptor and ligand [21]. At the moment no data are available for folate binding to C and B sites in *D. discoideum,* that discriminate between these two possible models. Ligand induced affinity changes seem to be a universal property of membrane bound receptors. It has been reported for β-adrenergic receptors [21–23], acetylcholine receptors [23,25] and muscarinic receptors [26] in higher organisms and for cAMP receptors in *D. discoideum* [11]. Probably these changes reflect the first steps in the pathway of signal transduction. Thus, a further investigation of the post-binding processes of folate receptors in *D. discoideum* might add to the general understanding of signal transduction across the plasma membrane.

Like the folate binding sites in this study, multiple oligopeptide receptor classes were also reported in leukocytes [12,13]. Furthermore, it was proposed that specific functions might be transduced by each receptor class. An intriguing question is, whether also in *D. discoideum* distinct responses are mediated by the different binding classes. In the accompanying report it is attempted to link one of the distinct binding classes to the chemotactic response [14].

Besides chemotaxis, folic acid also elicits cAMP synthesis and secretion. In recent reports methotrexate was observed to antagonize the folic acid induced cAMP response [27,28]. Presently, we are trying to elucidate the mechanism of this effect of methotrexate at the level of the binding sites.

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